

AD _____

GRANT NO: DAMD17-94-J-4319

TITLE: Analysis of Multistep Mammary Tumorigenesis in WNT-1 Transgenic Mice

PRINCIPAL INVESTIGATOR(S): Deepa Shankar, M.D.

CONTRACTING ORGANIZATION: Childrens Hospital Los Angeles
Los Angeles, California 90027

REPORT DATE: September 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

19951211 093

DTIC QUALITY INSPECTED 1

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 1995		3. REPORT TYPE AND DATES COVERED Annual : 1 Sep 94 - 31 Aug 95
4. TITLE AND SUBTITLE Analysis of Multistep Mammary Tumorigenesis in WNT-1 Transgenic Mice			5. FUNDING NUMBERS DAMD17-94-J-4319	
6. AUTHOR(S) Deepa Shankar, M.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Childrens Hospital Los Angeles Los Angeles, California 90027			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) <p>Mouse mammary tumor virus is used as a mutagen in transgenic mice that carry an activated <i>Wnt-1</i> gene, to produce additional events such as activation of a second oncogene by nearby insertion of proviral DNA. In about 55% of the mammary tumors having new proviral insertions, no known targets of MMTV mutations or known <i>Fgf</i> genes are affected, implicating the involvement of other proto-oncogenes. In order to identify one of the activated proto-oncogenes from this group of tumors, the cellular sequences flanking a new proviral insertion site from tumor 76 was cloned, the insert mapped, and repeat free fragments isolated. Southern blots of tumor DNAs probed with the cellular fragments show rearrangements due to MMTV proviral insertions within this locus in 12 of 85 tumors analyzed. These results indicate that this is a common insertion site for MMTV. Since northern analysis and exon trapping failed to identify an activated gene within the cloned region, the region downstream is being cloned and characterized. No known targets of MMTV mutations or members of the FGF family seem to be activated in these tumors. Therefore it is likely that we would find a novel or unexpected proto-oncogene activated in these tumors.</p>				
14. SUBJECT TERMS <p>Mouse mammary tumor virus (MMTV), <i>Wnt-1</i>, mammary tumors, transgenic mice, cooperating oncogenes, Breast Cancer</p>			15. NUMBER OF PAGES 12	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified 1	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to *stay within the lines* to meet *optical scanning requirements*.

Block 1. Agency Use Only (Leave blank).

Block 2. Report Date. Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

Block 3. Type of Report and Dates Covered. State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

Block 4. Title and Subtitle. A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

Block 5. Funding Numbers. To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C - Contract	PR - Project
G - Grant	TA - Task
PE - Program Element	WU - Work Unit Accession No.

Block 6. Author(s). Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

Block 7. Performing Organization Name(s) and Address(es). Self-explanatory.

Block 8. Performing Organization Report Number. Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es). Self-explanatory.

Block 10. Sponsoring/Monitoring Agency Report Number. (If known)

Block 11. Supplementary Notes. Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

Block 12a. Distribution/Availability Statement. Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

DOD - See DoDD 5230.24, "Distribution Statements on Technical Documents."

DOE - See authorities.

NASA - See Handbook NHB 2200.2.

NTIS - Leave blank.

Block 12b. Distribution Code.

DOD - Leave blank.

DOE - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.

NASA - Leave blank.

NTIS - Leave blank.

Block 13. Abstract. Include a brief (*Maximum 200 words*) factual summary of the most significant information contained in the report.

Block 14. Subject Terms. Keywords or phrases identifying major subjects in the report.

Block 15. Number of Pages. Enter the total number of pages.

Block 16. Price Code. Enter appropriate price code (*NTIS only*).

Blocks 17. - 19. Security Classifications. Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

Block 20. Limitation of Abstract. This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

PI In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

PI In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

PI In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI Signature 9/27/95
Date

TABLE OF CONTENTS

<u>PAGE</u>	<u>SECTION</u>
1	FRONT COVER
2	SF 298 REPORT DOCUMENTATION PAGE
3	FOREWORD
4	TABLE OF CONTENTS
5-6	INTRODUCTION
7-9	BODY OF REPORT
9	CONCLUSIONS
10-11	REFERENCES
12	APPENDIX

Accession For	
NTIS	CRA&J <input checked="" type="checkbox"/>
DTIC	TAB <input type="checkbox"/>
Unannounced	<input type="checkbox"/>
Justification	
By	
Distribution /	
Availability Codes	
Dist	Avail and/or Special
A-1	

II. INTRODUCTION

Breast cancer, like other cancers, results primarily from accumulation of genetic mutations. Many of the identified mutations associated with cancer result in the activation of proto-oncogenes or inactivation of tumor suppressor genes. In most cases, a single chromosomal aberration is insufficient to cause carcinogenesis but is rather the result of mutations in two or more genes. In order to understand the development and progression to cancer, it is imperative to identify not only the single mutations involved, but also synergistically acting groups of cancer related genes.

The role of retroviruses in viral-induced cancers has been well established in mice. For example, MMTV has proven to be a powerful tool for studying murine mammary tumorigenesis. MMTV is a B type retrovirus that is known to cause mammary adenocarcinomas in certain inbred strains of mice (e.g. C3H, BR6, GR) (1, 2). The tumor inducing property of MMTV is intrinsically related to an obligatory step in its life cycle, the insertion of a proviral copy of its genome into host cellular DNA. The integration is a mutagenic event for the host cells and as a consequence may lead to the transcriptional activation of closely linked proto-oncogenes by the mechanism of insertional mutagenesis (3). The activation of the proto-oncogene by MMTV contributes to the transformation of the cell and development of a tumor. A number of proto-oncogenes activated by MMTV in mammary tumors have been identified. They are *Wnt-1*, *Wnt-3*, *Wnt-10b*, *Fgf-3*, *Fgf-4*, and *Int-3*.

While the structure of these genes are known, less is known about their function. A common factor among the MMTV activated genes is that they all appear to play key roles in early embryonic development (2). To prove the oncogenic potential of *Wnt-1*, transgenic mice containing the *Wnt-1* gene under the control of an MMTV enhancer were generated. Both male and female transgenics developed mammary adenocarcinomas following a generalized mammary hyperplasia (21). The median latency of mammary tumor formation, in female mice was ~ 5 months. Males developed tumors less frequently and later in life. The generalized hyperplasia coupled with the long latency and the sporadic nature of the tumor formation suggest that *Wnt-1* contributes to but is not sufficient for mammary tumorigenesis in these mice. Activation of *Wnt-1* is probably an early event in the process of tumor formation. Therefore other events, presumably genetic, are necessary for tumor progression.

In an attempt to identify genes acting in synergy in the multistep process of murine mammary tumorigenesis, these *Wnt-1* transgenic mice were mutagenized by infection with MMTV (22, 23). The strategy was that since MMTV transcriptionally activates proto-oncogenes by insertion of its own DNA near them (2), one could possibly identify additional oncogenes that oncogenically cooperate with *Wnt-1* by tagging them with viral DNA. Activation of the cooperating oncogene would therefore confer a growth advantage and would presumably produce a tumor composed mainly of cells that are clonally derived from the cell bearing the proviral insertion. Implicit in this hypothesis was the expectation of a reduction in tumor latency. As predicted, in MMTV infected *Wnt-1* transgenics the median latency of tumor formation decreased from ~5 months to 2.5 months and the number of tumors per mouse increased (23). Southern blot data reveal that most of these tumors contained clonal tumor-specific proviruses in addition to the endogenous proviruses found in laboratory mice (23). The advantage of this approach over other mutagenesis procedures is that tumors arising due to proviral insertions contain proviruses physically linked to the activated proto-oncogenes forming a molecular tag which permits easy identification and cloning of the activated genes (2).

Analysis of the tumor DNAs derived from infected *Wnt-1* transgenic mice by Southern blotting showed that at least 80 of 128 tumors (59%) contained clonal MMTV-specific proviruses (23). These tumors were examined for the insertional activation of proto-oncogenes known to be activated by MMTV: *int-2/Fgf-3*, *hst/Fgf-4*, *int-3* and *Wnt-3* (2, 24). Approximately 45% of these tumors contained insertionally activated *int-2* and/or *hst* (23). These results show the cooperation of *int-2* and *hst* with *Wnt-1*, which strongly corroborates prior findings indicating the same cooperation (25,26). I (in collaboration with a Hem/Onc fellow Craig MacArthur) recently identified another member of the FGF family of growth factors that is insertionally activated by MMTV in 8 of 80 mammary tumors with clonal tumor-specific proviral insertions (27). This gene (*Fgf-8*) was cloned from one of the tumors that had a single tumor specific proviral insertion as described in the methods section. *Fgf-8* is transcriptionally activated in the tumors from a silent state(27). This is the third member of the FGF family to be activated in this system, indicating that *Fgfs* and *Wnts* are strong collaborators in inducing mammary tumors.

As we have already demonstrated, this infected *Wnt-1* transgenic system can be used to identify novel or/and unexpected oncogenes that are involved in mammary tumorigenesis, thereby demonstrating oncogenic cooperation with *Wnt-1* and elucidating the multiple steps involved in murine mammary tumorigenesis. We still have ~ 55% of the mammary tumors from infected *Wnt-1* transgenic mice with new proviral insertions in which the known targets of MMTV mutations are not affected. I had proposed to identify an insertionally activated gene(s) in tumors 47 and 76.

My specific aims:

1. Isolation and identification of proto-oncogenes (novel and unexpected) insertionally activated by MMTV in tumors of infected *Wnt-1* transgenic mice.
 - Identification of proviral-cellular junction fragments.
 - Clone cellular sequences flanking the proviral insertion
 - Locate and isolate the activated gene in the locus using Northern blot and exon trap strategies.
 - Determine the expression pattern of the gene in normal tissues and in tumors.
2. Characterization of the gene and analysis of the oncogenic potential of the identified proto-oncogene.
 - Demonstrate the oncogenic potential of the isolated proto-oncogene in cell culture transfection assays.
 - Demonstrate the gene's oncogenic potential *in vivo* using transgenic mice.
3. Demonstration of the cooperativity of *Wnt-1* with the proto-oncogene that is activated by MMTV.
 - Demonstrate cooperativity by cotransfection of C57MG cells.
 - Obtain definite proof of cooperativity by generating bitransgenic mice.

BODY OF ANNUAL REPORT

Isolation and identification of proto-oncogenes insertionally activated by MMTV in tumors from infected Wnt-1 transgenic mice.

Identification of Proviral-cellular junction fragment to be cloned:

The group of tumors that were known to have proviral insertions but no known activated gene were analyzed for the presence of a discrete clonal proviral-cellular junction fragment that could be cloned. Initial screening of the tumor DNAs using Southern blot analysis of EcoRV and Sst-I digested DNA samples showed proviral insertions within an apparently common locus in two independent tumors (tumors 47 and 76), as indicated by the common size of the MMTV-specific restriction fragments. MMTV integration into the host genome alters the restriction map of the locus because of the insertion of proviral DNA. Each clonal provirus will thus have different sized junction fragments depending on the restriction sites at the site of integration. Therefore, if there are proviral insertions from independent tumors within the same locus approximately at the same site (within a few hundred base pairs), then the sizes of the junction fragments will be the same with several enzymes and can be detected by hybridizing to MMTV-specific probes on Southern blots.

To confirm the initial observation made from the EcoRV and Sst I data, I made Southern blots of DNAs from these two tumors (47 and 76), digested with four different enzymes and probed them with a probe from the 5' end of MMTV(probe;gag). Junction fragments of similar sizes hybridizing to the MMTV probe were seen in each enzyme digest of these two tumor DNAs, but not of control DNA. Since the presence of proviral insertions within a common locus in two independent tumors is indicative of selection a particular phenotype (activation of a proto-oncogene), I decided to clone a junction fragment from Tumor 76.

Construction and Screening of a Subgenomic library from Tumor 76.

Fifty µg of tumor 76 DNA was digested with Sst-I and electrophoresed on an agarose gel. The 5.5 kb Sst-I junction fragment was size selected from the gel and purified using glass beads(Geneclean). The fragment was then cloned into lambda Zap II and packaged as phage using the Gigapack II kit (Stratagene). A subgenomic library (λ Zap 76)of ~ 1x10⁶ pfu of recombinant phage was generated. The library was probed with an MMTV LTR probe and 12 putative positive clones were isolated. These clones were subjected to two additional rounds of screening, and ultimately four clones were selected.

The newly cloned region was removed from λ Zap II by the process of *in vivo* excision; this process created a subclone of the cloned fragment contained in Bluescript phagemid (SS5.5). Next the subclone was restriction mapped using several single and double enzyme digests, and Southern blots. The entire subclone was screened for repeat sequences by hybridizing a southern blot containing restriction digests of the plasmid to

mouse genomic DNA(50ng), and two repeat free cellular fragments were identified: PP1.2 and XS0.8 (fig-1).

Identification of a common insertion locus for MMTV:

In order to look for insertions within the same locus in other tumors, the entire tumor panel was screened by Southern blotting using the two repeat-free cellular fragments as probes. Eighty five tumor DNAs were restriction digested with Xho-I, and EcoRV and Southern blotted. The resulting Southern blots were probed first with PP1.2(5') and then with XS 0.8(3') to look for rearranged or shifted restriction fragments. Rearranged bands were seen in 12 of 85 tumors with proviral insertions indicating that this was a common insertion locus for MMTV. These blots were stripped and reprobed with MMTV specific probes gag(5') and env(3') to determine the site and transcription orientation of the proviral insertions. These results were further confirmed by Southern blots of these 12 tumor DNAs digested with an additional three enzymes. A map of the locus with the location and orientation of MMTV integration was thus generated.

Exon trapping and Northern analysis:

In order to look for transcribed regions within the cloned cellular fragment, exon trapping procedure was performed. The 5.5 kb Sst-I fragment was cloned into the exon trap vector pML53In(30) in both orientations. Cos-7 cells were then transiently transfected with both the plasmids and the control vector by the Lipofectamine method (BRL). After transient transfection, RNA was made from the cells and reverse transcription polymerase chain reaction (RT-PCR) was performed using specific primers (27,30). Analysis of the PCR products showed only the 100 bp insulin fragment in all the lanes indicating that the splicing occurred between the 2nd and 3rd insulin exons (Control) but no exon was trapped from the cloned region.

To confirm this observation, I also did northern analysis on RNAs from some of the tumors that had proviral insertions within this locus. Both PP1.2 and XS 0.8 were used as probes for this analysis. No specific transcript was seen hybridizing to either of the probes. This confirmed the fact that I did not have any coding region within my cloned cellular region. Since the cloned region is only 4.5 kb and it is known that MMTV can activate genes even 10-20 kb away from the insertion (), I decided to do chromosome walking in order to identify the activated gene. The orientation of the MMTV proviruses within this locus seems to indicate that the activated gene may be downstream of the cloned region(fig). There seems to be two clusters of MMTV insertions, one upstream and one downstream (according to the map). I decided to clone the region between the two clusters (fig).

Cloning of the region downstream of SS 5.5:

Southern blot analysis of tumor 66 shows a Xho-I restriction fragment of 15 kb that hybridizes to both XS0.8 and MMTV gag. This tumor also has a fragment of 17 kb that hybridizes to MMTV env. This fragment will allow me to clone the region from the downstream cluster of insertions to the next Xho-I site. Cloning both these fragments would therefore give me approximately 23 kb of cellular DNA, and I should be able to locate the gene activated by these proviral insertions within this region easily.

I size selected the fragments as previously described, cloned them into Lambda Dash II vectors (Stratagene), and made two subgenomic libraries; Library-1 and 2. Library-1 was titered and screened using the XS0.8 and MMTV gag. I isolated four putative positives from library one, subjected them to two additional rounds of screening and ultimately two positives were selected. Lambda DNAs from the positive clones were made and I am in the process of generating restriction maps of the inserts. Library-2 has been titered and is currently being screened.

Once I have a good restriction map of the cloned region, I will perform the Exon trapping procedures using overlapping fragments spanning the entire region. The trapped fragment will then be analyzed as described in the methods section of the proposal. This locus is apparently a frequent site for MMTV proviral insertions in tumor DNAs (more insertions than Fgf-8) indicating that there is a very high probability of an activated proto-oncogene in the vicinity.

CONCLUSIONS:

I have successfully identified and cloned a proviral-cellular junction fragment from a tumor from MMTV-infected Wnt-1 transgenic mice. Using cellular probes derived from this cloned region I have found insertions in 12 of 85 tumors within this locus. The probability of the occurrence of proviral insertions within the same 30 kb locus in two independent tumors is only 10^{-10} (30). This result indicates that this is a new common insertion locus for MMTV. These tumors have been previously analyzed for the activation of known targets of MMTV activations and members of the FGF family(27). Since this so far seems to be a unique locus, it is likely that we would find a novel or unexpected proto-oncogene activated in these tumors. I am currently in the process of cloning and characterizing the region downstream of SS 5.5. I will soon search for activated gene(s) in this region using exon trap and Northern blot techniques. Once I have identified the gene, I will characterize it and prove its oncogenic potential as described in my proposal and statement of work.

VI. REFERENCES:

1. Nandi, S and C.M. McGarth (1973). Mammary Neoplasia in Mice. *Adv.Cancer Res.* 17: 353-414.
2. Nusse, R.(1991) Insertional Mutagenesis in Mouse Mammary Tumorigenesis. *Current Topics in Microbiology and Immunology.* 171: 43-63.
3. Kung, H.J., Boerkoel, C., and T.H. Carter. (1991). Retroviral mutagenesis of cellular oncogenes: A review with insights into the mechanisms of insertional activation. *Current topics in Microbiology and Immunology.* 171: 1-25.
4. Nusse, R. and H.E. Varmus (1982). Many tumors induced by mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* 31: 99-109.
5. Peters, G., S. Brookes, R. Smith, and C. Dickson (1983). Tumorigenesis by mouse mammary tumor virus: evidence for a common region for provirus integration in mammary tumors. *Cell* 33: 369-377.
6. Gallahan, D. and R. Callahan (1987). Mammary tumorigenesis in feral mice: identification of a new *int* locus in mouse mammary tumor virus (czech-II) induced mammary tumors. *J. Virol.* 61: 66-74.
7. Roelink, H.E. Wagenaar, S. Lopes Da Silva, and R. Nusse (1990). *Wnt-3* , a gene activated by proviral insertion in mouse mammary tumors is homologous to *int-1/Wnt-1* and is normally expressed in mouse embryos and adult brain. *Proc. Natl. Acad. Sci. USA* 87: 4519-4523.
8. Nusse, R., A. Brown, J. Papkoff, P. Scambler, G. Shackleford, A. McMahon , R. Moon and H.E. Varmus. (1991). A new nomenclature for *int-1* and related genes: The Wnt gene family. *Cell* 64: 231.
9. Papkoff, J.(1989). Inducible overexpression and secretion of *int-1* protein. *Mol. Cell. Biol.* 9: 3377-3384.
10. Shackleford, G. M. and H.E. Varmus(1987). Expression of the proto-oncogene *int-1* is restricted to post-meiotic male germ cells and the neural tube of mid-gestational embryos. *Cell* 50: 89-95.
11. Wilkinson, D.G., J.A. Bailes, and A.P. McMahon (1987). Expression of the proto-oncogene *int-1* is restricted to specific neural cells in the developing mouse embryo. *Cell* 50: 79-88.
12. Thomas, K.R., and M.R. Capecchi (1990). Targeted disruption of the murine *int-1* proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* 346: 847-850.
13. McMahon, A.P. and A. Bradley, (1990). The *Wnt-1* proto-oncogene is required for development of a large region of the mouse brain. *Cell* 62: 1073-1085.
14. Wilkinson, D.G., J.A. Bailes, and A.P. McMahon (1988) . Expression of the FGF related proto-oncogene *int-2* during gastrulation and neurulation in the mouse. *EMBO J.* 7: 691-695.
15. Jakobovits, A., G.M. Shackleford, H.E. Varmus, and Martin, G. (1986). Two proto-oncogenes implicated in mammary carcinogenesis, *int-1* and *int-2* , are independently regulated during mouse development. *Proc. Natl. Acad. Sci. USA* 83: 7806-7810.
16. McMahon, A. (1992). The *Wnt* family of developmental regulators. *TIGS* 8: 236-242.
17. Nusse, R. and Varmus, H.E (1992). *Wnt* genes. *Cell* 69: 1073-1087.
18. Gallahan, D., C. Kozak, and R. Callahan (1987). A new integration region (*int-3*) for mouse mammary tumor virus on chromosome 17. *J Virol.* 61: 218-220.
19. Robbins, J., B.J. Blondel, D. Gallahan, and R. Callahan (1992). Mouse mammary tumor gene *int-3*: a member of the notch gene family transforms mammary epithelial cells. *J. Virol.* 66: 2594-2599.

20. Adams, J. M., and S. Cory (1991). Transgenic models of tumor development. *Science* 254: 1161-1167.
21. Tsukamoto, A.S., R. Grosschedl, R.C. Guzman, T. Parslow, and H.E. Varmus (1988). Expression of the *int-1* gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. *Cell* 55: 619-625.
22. Shackleford, G. M., and H. E. Varmus. (1987) Construction of a clonable, infectious and tumorigenic mouse mammary tumor virus provirus and a derivative vector. *Proc. Natl. Acad. Sci. USA*, 85: 9655-9659.
23. Shackleford, G.M., C.A. MacArthur, H.C. Kwan, and H.E. Varmus. (1993). Mouse mammary tumor virus infection accelerates mammary carcinogenesis in *Wnt-1* transgenic mice by insertional activation of *int-2/ Fgf-3* and *hst/Fgf-4*. *Proc. Natl. Acad. Sci. USA*. 90: 740-744.
24. Peters, G., S. Brookes, R. Smith, and C. Dickson (1989). The mouse homolog of the *hst/k-Fgf* gene is adjacent to *int-2* and is activated by proviral insertion in some virally induced tumors. *Proc. Natl. Acad. Sci. USA*. 86: 5678-5682.
25. Peters, G., A.E. Lee, C. Dickson (1986). Concerted activation of two potential oncogenes in carcinomas induced by mouse mammary tumor virus. *Nature* 320: 628-631.
26. Kwan H. C., V. Pecenka, A. Tsukamoto, T.G. Parslow, R.Guzman, Lin, T.P., W.J. Muller, F.S. Lee, P. Leder, and H. E. Varmus (1992). Transgenes expressing the *Wnt-1* and *int-2* proto-oncogenes cooperate during mammary carcinogenesis in doubly transgenic mice. *Mol. Cell. Biol.* 12: 147-154.
27. MacArthur, C.A., D.B. Shankar, and Shackleford, G.M (1995). *Fgf-8*, activated by proviral insertion, cooperates with a *Wnt-1* transgene in mouse mammary tumorigenesis. *J. Virol*, 69: 2501-1507
28. Sambrook, J., E.F. Fritsch and T. Maniatis (1989). *Molecular cloning: A laboratory manual*, 2nd edition, (C. Nolen, ed.), Cold spring harbor laboratory press, Cold spring harbor, NY.
29. G. Peters (1990). Oncogenes at viral integration sites. *Cell growth and differentiation*. 1: 503-510.
30. D. Auch and M Reth (1990). Exon trap cloning: using PCR to rapidly detect and clone exons from genomic DNA fragments. *Nucleic Acids Res.* 18. 6743-6744.
31. Brown, A., R. Wilden, T. Prendergrast, H. E. Varmus. (1986). A retrovirus vector expressing the putative mammary oncogene *int-1* causes partial transformation of a mammary epithelial cell line. *Cell* 46: 1001-1009.
32. Sinn, E., W. J. Mueller, P.K., Pattengale, I Tepler, R. Wallace and P. Leder (1987). Co-expression of MMTV/ *v-Ha-ras* and MMTV/ *c-myc* in transgenic mice: synergistic action of proto-oncogenes *in vivo*. *Cell* 49: 465-479.
33. Hanahan, D. (1988). Dissecting multistep tumorigenesis in transgenic mice. *Ann. Rev. Genet.* 22: 479-519.
34. MacArthur, C.M., Shankar, D.B., Lawshe, A, Heikinheimo, M., and G. Shackleford (1995). FGF-8 isoforms differ in NIH3T3 Cell Transforming Potential. *Cell, Growth and Differ.* (in press).
35. Tuason, O., Shankar, D.B., and G. Shackleford. *Bek/FgfR2* a novel common insertion site for MMTV. (Unpublished).

(9) APPENDIX

A NEW COMMON INSERTION LOCUS FOR MMTV

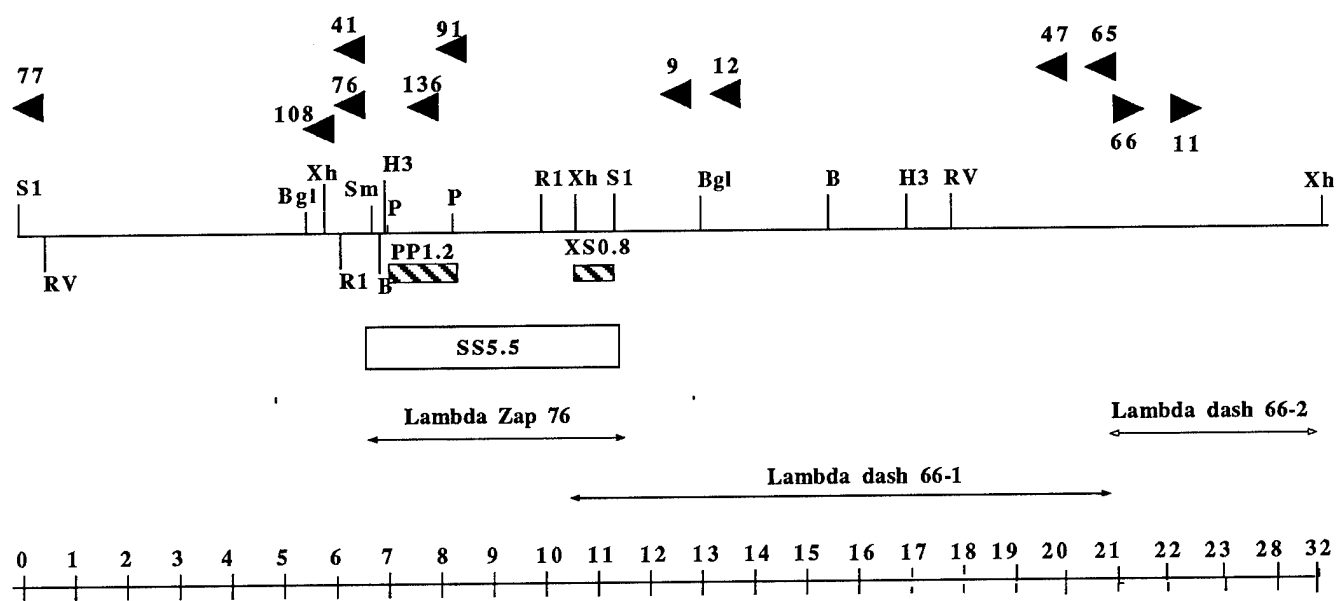


Figure-1: Map of the Tumor 76 locus . Arrowheads represent MMTV insertion sites and the orientation. R1 represent EcoR1, Bgl: Bgl II, B: Bam HI, P: Pst I, H3: Hind III, RV: EcoRV, S1: Sst I, Sm: Sma I, Xh: Xho I. Filled in boxes represent repeat free probes. Lines with arrowheads represent the Lambda clones.